Effects of chondroitin sulfate and interleukin-1β on human chondrocyte cultures exposed to pressurization: a biochemical and morphological study
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Summary

Objective: This study investigated the in vitro effects of chondroitin sulfate (CS) on human articular chondrocytes cultivated in the presence or in the absence of interleukin-1β (IL-1β) during 10 days of culture with and without pressurization cycles.

Design: The effects of CS (10 and 100 µg/ml) with and without IL-1β were assessed in the culture medium of cells exposed to pressurization cycles in the form of synusoidal waves (minimum pressure 1 Mpa, maximum pressure 5 Mpa) and a frequency of 0.25 Hz for 3 h by immunoenzymatic method on microplates for the quantitative measurement of human proteoglycans (PG). On the 4th and 10th day of culture the cells were used for morphological analysis by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Results: The presence of IL-1β determines a significant decrease in PG concentration measured in the culture medium. When the cells are cultured in the presence of IL-1β and CS, a statistically significant restoration of PG levels is observed. Under pressurization conditions, we observed that PG concentration in the medium of cells presents a significant increase at baseline conditions, in the presence of IL-1β+CS10 and IL-1β+CS100, but not with IL-1β alone. The results concerning metabolic evaluation are confirmed by the morphologic findings obtained by TEM and SEM.

Conclusions: These in vitro studies confirm the protective role of CS, which counteracts the IL-1β induced effects and they confirm the importance of pressure on chondrocyte metabolism and morphology. © 2000 OsteoArthritis Research Society International

Key words: Osteoarthritis, Chondrocytes, Pressurization system, Chondroitin sulfate.

Introduction

Osteoarthritis (OA) is one of the most common rheumatic diseases directly involving the articular cartilage. The biomechanical properties of the cartilage depend upon the extracellular matrix produced by the chondrocytes, cells which are capable of maintaining a dynamic equilibrium between the anabolic and catabolic processes.¹ The metabolic activity of these cells is regulated by several mediators, such as cytokines, hormones and growth factors.²,³ Interleukin 1 (IL-1) is a cytokine involved in cartilage degradation processes.⁴⁻⁸ It is produced by several types of cells and can be found in the synovial fluid of osteoarthritic subjects.⁹,¹⁰

The metabolic activity of chondrocytes is further influenced by mechanical factors depending on the articular load.¹¹,¹² At rest the hydrostatic pressure on the articular cartilage is about 2 atm; however, during ordinary physical activity it can increase to 100–200 atm.¹²

Several in vitro studies have shown the importance of articular load as a modulator of cartilage metabolism. In fact, the articular load influences the concentration of proteoglycans (PG) and cations as well as the osmolarity of the cartilage microenvironment.¹³,¹⁴ These studies show that chondrocytes respond to intermittent loading by increased PG synthesis, whereas continuous mechanical loading produces the opposite effect. The response is further modified by the magnitude, duration and frequency of loading.¹⁵⁻¹⁷ Hydrostatic pressure affects proteins, the cytoskeleton and cell organelles; compression of cartilage results in the deformation of cells and the extracellular matrix, as well as physicochemical changes which include altered matrix water content, fixed charge density, mobile ion concentrations and osmotic pressure.¹¹,¹³⁻¹⁷

The way in which loading is related to the incidence of OA is not clear, although some studies have shown that low levels of intermittent fluid pressure, such as those that occur in vivo during ilizarov joint distraction,¹⁸ have beneficial effects on joint tissue in OA, indicating that this factor could be useful in the treatment of OA.¹⁹,²⁰

Current OA treatment includes physical, pharmacological and surgical approaches. Pharmacological treatments have been divided into three categories: (1) analgesic and nonsteroidal anti-inflammatory drugs (NSAIDs); (2) symptomatic slow-acting drugs for OA; and (3) disease modifying drugs.²¹ The drug investigated in this study, sodium chondroitin sulfate (CS), falls under the second category.²¹ CS is a glycosaminoglycan which is naturally found in the extracellular matrix of articular cartilage and is composed of
a long unbranched polysaccharide chain with a repeating disaccharide structure of N-acetylgalactosamine and glucuronic acid. The benefits of the symptomatic effects of CS in the treatment of OA have been tested in several clinical trials.23–26

Chondrocyte cultures represent a valid and simplified biological model in which to test the effects of drugs and such agents as cytokines, hormones and growth factors. They can be used also for the evaluation of the influence of hydrostatic pressure on chondrocyte morphology and metabolism.

In this work we have studied the in vitro effects of CS (Condrosulf®, IBSA Switzerland) on cultures of human articular chondrocytes cultivated in the presence or in the absence of IL-1β, during 10 days of culture with and without pressurization cycles. Under these conditions we evaluated the effect of CS±IL-1β through metabolic (PG levels in the culture medium) and morphologic assessments carried out with a transmission electron microscope (TEM) and a scanning electron microscope (SEM).

Materials and methods

The chondrocytes used in the study were grown in alginate gel following previously defined techniques.27,28 Alginate is a linear polysaccharide extracted from brown marine seaweed, composed of 1–4 linked β-D-mannuronic acid and α-L-guluronic acid.28,29 In our study we used a 0.75% alginate solution that had been sterilized previously in an autoclave at 120°C for 20 min. In the presence of bivalent cations, particularly Ca2+, alginate polymerizes to form a semi-solid support that can be used in the culture and growth of chondrocytes. If necessary, the gel can easily be dissolved and the cells can be used for morphologic and biochemical evaluations.

Chondrocyte extraction from human articular cartilage

Human articular cartilage was obtained from the femoral heads of six OA subjects undergoing surgery for total hip prostheses (mean age±SD of the patients: 67.5 years±2.51, range 64–70 years). Immediately after surgery, macroscopically healthy cartilage were cut aseptically and minced into 2-mm² pieces. The cartilage fragments were washed in saline solution (A) (in mM: 140 NaCl, 5 KCl, 5 glucose, 10 HEPES, pH 7.4) containing 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM glutamine and 50 μg/ml ascorbate in Dulbecco’s minimum essential medium (DMEM) were added. The Petri dishes were maintained in an atmosphere of 5% CO₂ in air at 37°C and the culture medium was changed every 2 days.

Study with chondroitin sulfate

The effects of CS±IL-1β were analysed on chondrocytes cultured as described above and the cultures were inoculated in Petri dishes (0.35 mm) at a density of 5×10⁵ cells/ml.

The CS (25 KDa) was extracted from bovine cartilage. Various analytical methods were used to demonstrate the high degree of purity of the product (>97%). CS was extracted and purified by treatment with proteolytic enzymes and precipitation in the presence of organic solvents. Electrophoretic separations of CS in agarose-gel and in Titan III did not detect contaminant glycosaminoglycans containing uronic acids. Less than 2% proteins or peptides were detected by Lowry assay.32 Less than 0.5 (w/w) nucleic acids were measured in the preparation of the CS.

The CS (0, 10, 100 μg/ml), with or without human recombinant IL-1β (Boehringer Mannheim, Italy) 5 ng/ml, was added to the culture medium throughout the test period. Every 2 days the medium was removed and stored at −70°C pending determination of its PG content.

Pressurization system

Our pressurization system is different, in some aspects, from most of those currently in use.33 The pressure chamber is a hermetically sealed stainless steel cylinder with a height of 400 mm and an internal diameter of 90 mm. Chondrocyte cultures in Petri dishes are filled with culture medium, well sealed with a special membrane and then placed inside the pressure chamber. The chamber is filled with distilled water and maintained by a thermostat at a constant temperature of 37°C. The chamber is then pressurized by the oleodynamic energy produced by an electric relay and applied, by means of a transfer accumulator, to the water in the chamber. The operator can pre-set and change the pressure inside the chamber during the test with a data gathering and processing system installed in a PC and based on turbo Pascal language. The cells used in our tests were treated as described above. The Petri dishes were sealed with a Surlyn 1801 Bynel CXA 3048 bilayer membrane (thickness 90 μm; Du Pont, Italy) after excluding all air. The air in the dishes must be removed to avoid implosions due to the presence of air which remains between the membrane and the medium in the Petri dishes. Surlyn membrane is partly permeable to O₂ and CO₂, but not to water or its other solutes. The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive (3M, Italy). The chondrocytes covered in culture medium were then pressurized according to sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 5 MPa and a frequency of 0.25 Hz. The cells
were pressurized for 3 h every 2 days (days 2, 4, 6, 8 and 10). After the pressurization phase, the medium was collected and stored at −70°C for PG determination. On the 4th and the 10th days of culture some dishes were used for morphological analysis. After pressurization, the cells in alginate gel were immediately fixed for transmission electron microscopy (TEM) and for scanning electron microscopy (SEM). Dishes that were not pressurized served as controls.

BIOCHEMICAL ASSAYS

The quantity of PG in the culture medium was measured at different intervals (2, 4, 6, 8 and 10 days) by immunoenzymatic method on microplates for the quantitative measurement of human PG (Medgenix Diagnostics, Belgium). This technique uses two monoclonal antibodies which are directed against the PG’s keratan sulfate, and another monoclonal antibody which is directed against the bonding site of the PG’s hyaluronic acid. Standards and samples containing PG react with the monoclonal antibody (M Abs1) attached to the well of the microplate, as well as with another monoclonal antibody (M Abs2) labelled with horse-radish peroxidase (HRP). After an incubation period which allowed for the formation of a sandwich made up of coated M Abs1/PG/M Abs2-HRP, the wells were washed several times in order to remove the labelled antibody which had not bonded. The amount of bonded antibody was measured by adding and incubating a chromogen solution. The reaction was then blocked by adding a stop solution. The microplate was read at 450 nm and the quantity of substrate was determined colorimetrically by measuring the absorbance, which is proportional to PG concentration. The assay sensitivity was 0.9 ng/ml.

The DNA content in culture was analysed according to Labarca and Paigen’s fluorometric method. This method is based on the development of a fluorescent emission when the fluorochrome-bis-benzimidazol reagent (Hoechst dye 33258) bonds with the DNA in culture.

TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

The cells were processed for morphological analysis on the 4th and the 10th days of culture. For transmission electron microscopy, all samples were fixed for 2 h at 4°C in cold Karnovsky fixative, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 1 h at 4°C in 1% buffered OsO4, dehydrated in a graded series of ethanol and embedded in Epon-Araldite. Ultrathin sections cut with an LKB III ultramicrotome were collected in copper grids, stained with uranyl acetate and lead citrate and then photographed with a Philips CM10 electron microscope. We observed at least 50 cells from each group for evaluation.

For scanning electron microscopy, the cells in alginate were fixed for 3 h at 4°C in Karnovsky fixative, washed in cacodylate buffer 0.1 M at 4°C overnight, post-fixed in 1% OsO4 in veronal acetate buffer for 2 h and, after a brief wash in cacodiylate buffer 0.1 M, placed in citrate pH 7.4 to remove the alginate. The chondrocytes were dehydrated in a graded series of ethanol, placed in tert-butanol and frozen at 0°C before drying by sublimation of the tert-butanol in a vacuum chamber. The samples were sputter coated with gold and observed in a Philips SEM 505. We observed at least 100 cells from each group for evaluation.

STATISTICAL ANALYSIS

The data were expressed as the mean±SD of PG release into the culture medium per micrograms of DNA in the six tested cultures. The Student’s t-test was used for the statistical analysis: P<0.05 was considered significant. When data were not normally distributed, the Mann–Whitney U test was used.

Results

The total PG concentration in the culture medium during the 10 days of culture (pooled data from days 2, 4, 6, 8, 10) at baseline conditions, in the presence of a damaging stimulus represented by IL-1β at a concentration of 5 ng/ml and in the presence of IL-1β and of CS at the two concentrations used in the study, i.e. 10 µg/ml and 100 µg/ml of culture medium, is shown in Fig. 1. The presence of IL-1β determines a significant decrease (P<0.05) in PG levels, but when the cells are cultured in the presence of IL-1β and CS a statistically significant restoration (P<0.05) of PG concentration is observed.

In Fig. 2 we have compared the data shown in Fig. 1 with those obtained under pressurized conditions. As Fig. 2 shows, there is a significant increase (P<0.01) in the level of PG in the culture medium in basal conditions. However, with pressurization there is a slight and insignificant increase in the concentration of PG in the presence of IL-1β. In pressurized conditions, the addition of CS induces a significant increase (P<0.005 per CS10 µg/ml and P<0.003 per CS100 µg/ml) of PG in the culture medium.

The levels of PG in the culture medium at the different times and in the different experimental conditions is demonstrated in Table I. With the passage of time, there was a progressive but not significant decrease in the concentration of PG in the culture medium.

The morphological evaluations, obtained by TEM and SEM, seem to indicate an effect of IL-1 of the CS and of the pressure on the metabolic functions of the chondrocytes. Figure 3 shows chondrocytes photographed on the fourth day of culture by TEM. Figure 3A shows a cultured cell at basal conditions: the nucleus appears euchromatic, the cytoplasm contains a fair amount of rough endoplasmic reticulum and lipid droplets, and the cytoplasmic processes of the plasma membrane extending out into the surrounding matrix have abundant collagen fibres and PG granules. Figure 3B shows a cell cultured in the presence of IL-1β and its damage is evident: several vacuoles in the cytoplasm are devoid of typical structures. The extracellular matrix lacks both collagen fibers and PG granules. Figure 3C shows a cell cultured in the presence of IL-1β+CS100 µg/ml and a clear restoration of the cell structures can be observed: the nucleus shows a distinct nucleolus, the cytoplasm contains rough and smooth endoplasmic reticuli, a Golgi complex and lipid droplets. Figure 3D shows a cell cultured at basal conditions and subjected to pressurization: the abundant presence of collagen fibers and PG granules in the extracellular matrix are evident.

Figure 4 shows the SEM image of cells photographed on the 4th day of culture. Figure 4A shows a cell cultured at baseline conditions: it is interesting to observe that it has retained its spherical shape, some secretion granules and the thick network of collagen fibrils. Figure 4B shows a cell cultured in the presence of IL-1β: its damage is clearly evident and this is further confirmed by the loss of cytoplasmic processes and by the presence of superficial...
alterations. Figure 4(C) shows a cell cultured in the presence of IL-1β+CS100 µg/ml; the cell morphology has been partially restored as confirmed by the presence of several surface granules. Figure 4D shows a cell cultured at basal conditions and subjected to pressurization: its SEM image shows the presence of abundant matrix fibers and secretion granules. Figure 4E shows a cell cultured in the presence of IL-1β and subjected to pressurization: its damage is shown in the absence of granules and the lack of a network of collagen fibers, although its characteristic spherical shape appears slightly restored. Figure 4F shows a cell cultured in the presence of IL-1β+CS100 µg/ml and subjected to pressurization; it shows the presence of abundant extracellular matrix fibers and the restoration of the cell surface morphology.

Figure 5 shows chondrocytes photographed on the 10th day of culture by TEM. Figure 5A shows a cultured cell at baseline conditions. The ultrastructure is still as well conserved as it was on the 4th day of culture. Figure 5B shows a cell cultured in the presence of IL-1β+CS100 µg/ml and subjected to pressurization: the cell shows a similar morphology on the 4th and the 10th days of culture.
Discussion

In our study we have used alginate as the support in the culturing of human chondrocytes in considering the advantages and drawbacks of most currently used techniques. Alginate gel is a valid alternative to other culture techniques since it enables the chondrocyte to retain its 3D-structure and to maintain its characteristic cell...
shape, while preventing cell dedifferentiation.\textsuperscript{27,28} The chondrocytes cultured with this technique are in a condition which is the most similar to the \textit{in vivo} situation and are therefore valuable in drawing reliable morphological and metabolic assessments.\textsuperscript{39–41}

In this study we have tested the effects of IL-1\textbeta and CS in the presence and the absence of pressurization cycles on the morphology and metabolism of \textit{in vitro} human chondrocytes. These tests confirm the studies already carried out on \textit{in vitro} experimental models.\textsuperscript{39,42–44}

It is currently accepted that IL-1 is an important mediator in cartilage destruction. IL-1 is a prime up-regulator of metalloproteinase (MMP) gene expression by chondrocytes, and it also down-regulates the production of the endogenous inhibitor of MMPs, the TIMPs as well as the synthesis of PG and collagens.\textsuperscript{45}

Our experiments have shown that the addition of IL-1 determines a reduction of the concentration of the PG in the culture medium; this could be the result of the fact that cytokine induces an inhibition of PG synthesis by the chondrocytes, as shown in the morphological analysis. The morphological aspects show signs of cellular suffering with the presence of vacuoles and the lack of cellular organelles typically responsible for the synthesis of the matrix glycoproteins (the endoplasmic reticulum, the Golgi apparatus and mitochondria) (Figs 3B and 4B).

When the cells were cultivated in the presence of IL-1\textbeta+CS there was a restoration of PG concentration in the culture medium. This fact confirms the protective role played by this substance which counteracts the IL-1\textbeta induced effects and might be used by the chondrocyte as a substratum for the synthesis of PG.\textsuperscript{46}

The articular cartilage is a tissue constantly subjected to a load which depends upon body weight and muscular tension. The load also varies according to posture and physical activity. Hydrostatic pressure is one of the several factors which operate in articular cartilage subjected to loading.\textsuperscript{11,13–17,47,48} Hydrostatic pressure appears to
modulate aggrecan biosynthesis through membrane-mediated pathways, such as the transport of cations, amino acids and macromolecules. It has also been suggested that hydrostatic pressure may alter the action of the membrane Na+/K+ pump, thus altering intracellular K+ concentrations.49

In our study, the pressure values applied were within the physiological range of human joints. In fact, pressure peaks of up to 18 MPa have been measured in human hip joints; however, pressure levels of 5 MPa are most often encountered in the knee joint during normal gait.12,50

Chondrocytes undergoing physiological pressurization according to sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 5 MPa and a frequency of 0.25 Hz presented a greater metabolic activity which was expressed in the increase of PG levels in the culture medium at basal conditions (Fig. 2). This fact was also confirmed in morphological analysis by TEM and SEM (Figs 3D and 4D). In our opinion the increase of PG in the culture medium, which was also observed in basal conditions, could be determined by a stimulation of cellular activity induced by the pressurization. Relatively low hydrostatic pressures have been shown to determine increases in cyclic AMP and PG synthesis and decreases in DNA synthesis.51 However, the signal transduction mechanisms that lead to these metabolic changes must still be clarified. It is not clear that higher pressures, within the physiological range, like those used in the present study, operate by the same mechanism. The stimulating effect of the physiological hydrostatic pressure which we have shown is in agreement with that found by other authors who have also utilized human osteoarthritic cartilage. Some studies have shown that OA chondrocytes are more sensitive to physiological hydrostatic pressure than normal chondrocytes.19,20,52 The ‘stimulating’ effect of the pressure did not, however, manage to counterbalance the negative effects determined by the addition of IL-1β which, in fact, induce a serious metabolic and morphological imbalance.

However, the addition of CS created a protection from the effects of IL-1β under the pressurization conditions used in the study. The simultaneous use of pressurization with the presence of CS in fact determined a highly significant increase in PG concentration, as if a synergism of action exists between the drug and the mechanical factor regarding IL-1β. The biochemical data was also supported by morphological analysis (Figs 4F and 5B).

The decrement in the release of PG in the culture medium over time could be explained, in our opinion, by the entrapment of the PG in the cellular clusters. This assertion, in agreement with the findings of other authors,53,54 was confirmed in the morphological analysis by TEM on the 10th day of culture. Figure 5 shows morphologically and ultrastructurally well preserved cells that are organized in a way that is similar to those observed on the 4th day of culture.

In conclusion, the study confirms the importance of pressure on chondrocyte metabolism. When subjected to pressure, the cells behave differently towards both damaging and protective stimuli and this fact further supports the necessity of studying these cells in vitro by recreating a situation that imitates as nearly as possible the in vivo conditions. Studies using chondrocyte cultures undergoing pressurization represent the most suitable experimental models for understanding both the etiopathogenetic mechanisms of OA and which type of physical activity may be best suited for the prevention of OA and for its therapy.

Our study points out the protective effects of CS on IL-1β in basal conditions and after pressurization of the chondrocytes. These results confirm in vitro the data obtained in clinical studies on patients with OA23–26 and on experimental animal models of OA.55

Further studies are necessary in order to clarify the mechanism of action of this drug and for the better understanding of the importance of this approach in OA therapy.

References
